Rapid Publication

Effect of Oral Creatine Supplementation on Human Muscle GLUT4 Protein Content After Immobilization

B. Op 't Eijnde, B. Ursø, E.A. Richter, P.L. Greenhaff, and P. Hespel

The purpose of this study was to investigate the effect of oral creatine supplementation on muscle GLUT4 protein content and total creatine and glycogen content during muscle disuse and subsequent training. A double-blind placebo-controlled trial was performed with 22 young healthy volunteers. The right leg of each subject was immobilized using a cast for 2 weeks, after which subjects participated in a 10-week heavy resistance training program involving the knee-extensor muscles (three sessions per week). Half of the subjects received creatine monohydrate supplements (20 g daily during the immobilization period and 15 and 5 g daily during the first 3 and the last 7 weeks of rehabilitation training, respectively), whereas the other 11 subjects ingested placebo (maltodextrine). Muscle GLUT4 protein content and glycogen and total creatine concentrations were assayed in needle biopsy samples from the vastus lateralis muscle before and after immobilization and after 3 and 10 weeks of training. Immobilization decreased GLUT4 in the placebo group (–20%, $P < 0.05$), but not in the creatine group (+9% NS). Glycogen and total creatine were unchanged in both groups during the immobilization period. In the placebo group, during training, GLUT4 was normalized, and glycogen and total creatine were stable. Conversely, in the creatine group, GLUT4 increased by ~40% ($P < 0.05$) during rehabilitation. Muscle glycogen and total creatine levels were higher in the creatine group after 3 weeks of rehabilitation ($P < 0.05$), but not after 10 weeks of rehabilitation. We concluded that *1*) oral creatine supplementation offsets the decline in muscle GLUT4 protein content that occurs during immobilization, and *2*) oral creatine supplementation increases GLUT4 protein content during subsequent rehabilitation training in healthy subjects. *Diabetes* 50:18–23, 2001

From the Faculty of Physical Education and Physiotherapy (B.O.E., P.H.), Exercise Physiology and Biomechanics Laboratory, Katholieke Universiteit Leuven, Leuven, Belgium; the Department of Human Physiology (B.U., E.A.R.), Copenhagen Muscle Research Center, University of Copenhagen, Copenhagen, Denmark; and the School of Biomedical Sciences (P.L.G.), Queens Medical Center, University of Nottingham, Nottingham, U.K.

Address correspondence and reprint requests to Peter Hespel, PhD, Faculty of Physical Education and Physiotherapy, Exercise Physiology and Biomechanics Laboratory, Tervuursevest 101, B-3001 Leuven, Belgium. E-mail: peter.hespel@flok.kuleuven.ac.be.

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AMPK, AMP-activated protein kinase; CK, creatine kinase; DW, dry weight; MAPK, mitogen-activated protein kinasE; RM, repetition maximum.

It is well established that high-dose (20–25 g per day) oral creatine intake can rapidly (3–5 days) raise muscle total creatine content. This elevation in muscle creatine storage is associated with increased muscle power output during short high-intensity exercise. In addition, it has been shown that long-term creatine intake can enhance the effects of weight training on muscle volume and strength (1,2). The use of creatine as an ergogenic supplement in sports has prompted interest in the potential of oral creatine supplementation to treat muscle atrophy and neuromuscular diseases. Thus, the recovery of muscle disuse atrophy due to immobilization was significantly enhanced by creatine supplementation (P.H., B.O.E., M. Van Leemputte, B.U., P.L.G., V. Labarque, S. Dymarkowski, P. Van Hecke, E.A.R, unpublished observations). Furthermore, creatine supplementation was found to have a beneficial impact on muscle functional capacity in various modes of mitochondrial cytopathies (3) and muscle dystrophies (4). At the same time, evidence is accumulating to suggest that creatine supplementation may be an effective neuroprotective agent to treat neurodegenerative diseases (5–7).

Interestingly, a number of recent observations also indicate that creatine supplementation might have a beneficial impact on glucoregulation. For instance, it has been shown that the ingestion of creatine in combination with carbohydrate supplements can stimulate postexercise muscle glycogen resynthesis (8), which is conceivably due to enhanced insulin-mediated muscle glucose uptake (9). Similarly, creatine intake in conjunction with carbohydrates was found to result in greater muscle creatine accumulation than creatine intake alone (10), which may be due to the fact that both glucose transport and creatine transport (11) in muscle cells are stimulated by insulin. On the other hand, a number of in vitro studies have found that high extracellular concentrations of guanidine compounds, including creatine, stimulate pancreatic insulin secretion (12,13). However, the extracellular creatine concentrations obtained by oral creatine intake in humans do not affect insulin secretion (14,15). Perhaps the most striking evidence to suggest that creatine supplementation might be an effective strategy to treat insulin resistance comes from a recent study on transgenic Huntington mice. The addition of creatine to the diet of the Huntington mice resulted in a marked neuroprotective effect and significantly reduced the hyperglycemia typical of these mice, while improving the glucose response to intravenous glucose injection (5).

Based on the above evidence, we speculate that creatine supplementation may enhance insulin-mediated muscle glucose uptake and glycogen synthesis, thereby beneficially impacting whole-body glucose homeostasis. This creatine

response might be particularly relevant to the prevention and/or treatment of disease states characterized by peripheral insulin resistance, such as type 2 diabetes, obesity, and inactivity (16). Furthermore, it is well established that muscle inactivity and training are effective stimuli to down- and upregulate muscle GLUT4 content and peripheral insulin sensitivity, respectively (17). Therefore, we investigated the effect of creatine supplementation on muscle GLUT4 protein content and total creatine and glycogen concentration in healthy volunteers during 2 weeks of leg immobilization and during 10 weeks of subsequent rehabilitation training. This report is part of a larger study (P.H., B.O.E., M. Van Leemputte, B.U., P.L.G., V. Labarque, S. Dymarkowski, P. Van Hecke, E.A.R) that investigated the effects of creatine supplementation on muscle functional capacity during disuse atrophy in healthy subjects.

RESEARCH DESIGN AND METHODS

Subjects. A total of 13 men and 9 women, aged 20–23 years, gave their informed written consent to participate in the study. They were instructed to abstain from taking any medication and to avoid making any changes in their usual physical activity level and other living habits during the period of the study. However, three of the women were taking oral contraceptives for the duration of the study. The local ethics committee approved the study protocol.

Study protocol. A double-blind study was performed over a 12-week period. At the start of the study, subjects were systematically assigned to either a creatine or a placebo group based on quadriceps muscle cross-sectional area and maximal isometric knee-extension torque to obtain two groups of similar distribution (P.H., B.O.E., M. Van Leemputte, B.U., P.L.G., V. Labarque, S. Dymarkowski, P. Van Hecke, E.A.R, unpublished observations). After baseline measurements had been taken, a light polyester cast, extending from groin to ankle, immobilized each subjects' right leg at a knee angle of ~160° for 2 weeks. Thereafter, the cast was removed, and the subjects underwent a standardized 10-week rehabilitation program. Each training session consisted of four series of 12 unilateral knee-extensions on a knee-extension apparatus, at a workload of 60% of maximal isometric knee-extension torque and at a rate of three sessions per week. Maximal knee-extension torque was measured at a 90° knee-angle at the start of each session using a calibrated force transducer. During the final 7 weeks of the training period, the series of four unilateral knee-extensions was increased to six. All training sessions were supervised by one of the investigators. During immobilization, the creatine group received 5 g creatine monohydrate four times per day, whereas the placebo group received placebo supplements (5 g maltodextrine, four times per day). During the training period, creatine and placebo supplementation was reduced to 5 g three times per day from week 1 to 3 and then to a single 5-g daily dose from week 4 to 10. The creatine supplements were flavored by the addition of citrate (60 mg/g creatine) and maltodextrine (940 mg/g creatine), whereas the placebo group ingested maltodextrine containing citrate (40 mg/g maltodextrine). Creatine and placebo powders were identical in taste and appearance. Before and after 2 weeks of immobilization, and after 3 and 10 weeks of rehabilitation, a percutaneous needle biopsy from the vastus lateralis muscle was obtained. The last training session preceded muscle sampling by at least 48 h. In addition, the subjects received a standardized dinner (855 kcal, 47% carbohydrate, 25% fat, and 28% protein) the evening before and a standardized breakfast (320 kcal, 65% carbohydrate, 15% fat, and 20% protein) the morning of muscle sampling. To collect each muscle biopsy, an incision was made through the skin and muscle fascia under local anesthesia (2–3 ml 1% lidocaine). During sessions 2–4, the incision was made either proximal or distal to the incision made at an earlier session. On removal from the limb, a piece of each muscle biopsy was immediately blotted and cleaned from visible connective tissue, rapidly frozen in liquid nitrogen, and stored at –80°C for subsequent biochemical and immunochemical analyses.

Biochemical and immunochemical analyses. The biopsy samples were first freeze-dried, then washed twice in petroleum ether to remove fat, and finally dissected free of the remaining visible blood and connective tissue. A fraction of each sample was pulverized, and the powdered extracts were used for spectrophotometric determination of glycogen and free creatine and phosphocreatine concentrations (18). Another fraction was used for GLUT4 determination. An aliquot of the freeze-dried muscle was homogenized (Polytron) for 30 s on ice in a buffer with the following composition: 150 mmol/l NaCl, 1% NP_4O, 0.5% deoxycholate, 0.1% SDS, and 50 mmol/l Tris, pH 8. The homogenate was incubated on ice for 1 h and spun for 15 min at 13,000*g*, and the supernatant (extract) was collected for analysis. Then, 100 µg of the extract were resolved by SDS-PAGE before electroblotting to polyvinylidine fluoride membranes. GLUT4 proteins were then detected by incubation in Tris-buffered saline with Tween (150 mmol/l NaCl, 50 mmol/l Tris, and 0.1% Tween 20) after blocking in 1% bovine serum albumin with a specific goat polyclonal antibody against the 13 COOH-terminal amino acids of GLUT4. Finally, GLUT4 was visualized by an alkaline phosphatase-labeled antibody and quantified on a phosphoimager (STORM; Molecular Dynamics, Sunnyvale, CA).

Data analysis. Data are means ± SE. Muscle total creatine concentration was calculated as the sum of free creatine and phosphocreatine. Treatment effects (creatine versus placebo) were evaluated by a two-way analysis of variance, which was covariate adjusted for the baseline values (Statistica; Statsoft, Tulsa, OK). In addition to these primary analyses, we did a one-way analysis of variance to compare the values after immobilization and rehabilitation with the corresponding baseline values within each group. The statistical analyses of the GLUT4 data were performed on the raw data (densitometric counts). $P < 0.05$ was considered statistically significant.

RESULTS

Muscle GLUT4 content. Muscle GLUT4 concentrations were expressed relative to the corresponding baseline values that were set equal to 1 (Fig. 1). Muscle GLUT4 content at baseline was similar between the groups. In the placebo group, 2 weeks of immobilization decreased GLUT4 content on an average of 22% (range –10 to –35%, $P < 0.05$). Conversely, in the creatine group, muscle GLUT4 protein was stable (+9% NS). In the placebo group, the rehabilitation training restored muscle GLUT4 content within 3 weeks to the baseline value, where it remained. However, in the creatine group, muscle GLUT4 content progressively increased during the 10-week rehabilitation period to a value that was ~40% higher than in the placebo group at the end of the study ($P < 0.05$).

Muscle glycogen. The initial muscle glycogen concentration was 407 ± 43 mmol/kg dry weight (DW) in the placebo group versus 379 ± 19 mmol/kg DW in the creatine group (NS) (Fig. 2). Immobilization did not change muscle glycogen concentration in either group. However, during the initial 3 weeks of rehabilitation training, muscle glycogen markedly increased in the creatine group ($P < 0.05$), whereas it did not significantly change in the placebo group. Thus, after 3 weeks, muscle glycogen concentration was higher ($P < 0.05$) in the creatine group (660 ± 70 mmol/kg DW) than in the placebo group (520 ± 60 mmol/kg DW). However, during the final 7 weeks of rehabilitation training, muscle glycogen reverted to baseline values in both groups.

Muscle creatine content. The muscle phosphocreatine and free creatine concentrations at baseline were similar between both groups (Table 1). During immobilization, phosphocreatine concentration decreased to ~15% below the baseline value in the placebo group ($P < 0.05$). This decrease was negated by creatine supplementation ($P < 0.05$). In the placebo group, muscle phosphocreatine concentration returned to the preimmobilization baseline level within the initial 3 weeks of the rehabilitation period, after which it remained stable. On the other hand, in the creatine group, compared with the placebo group, the muscle phosphocreatine concentration increased to ~12% above baseline value after 3 weeks of rehabilitation ($P < 0.05$). However, this increase above baseline in phosphocreatine was reversed during the final stage of the rehabilitation period. Throughout the study, the muscle free creatine concentrations were not significantly different between the placebo and the creatine groups. In the placebo group, muscle total creatine concentration was not significantly changed compared with the baseline value during either immobilization or rehabilitation. Yet

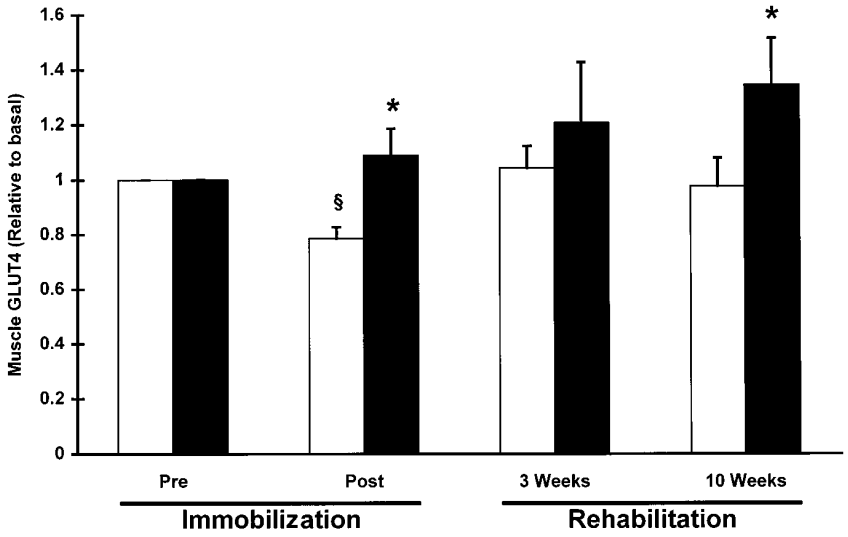

FIG. 1. Effect of creatine supplementation on muscle GLUT4 protein content during immobilization and subsequent rehabilitation training. Data are means ± SE (n = 8) and are expressed relative to the baseline value that was set to be equal to 1. Muscle samples were taken from the vastus lateralis muscle before and after 2 weeks of immobilization and after 3 and 10 weeks of rehabilitation of the right leg. During immobilization and rehabilitation, subjects ingested creatine monohydrate (■) or placebo (□). See RESEARCH DESIGN AND METHODS for further details. *Significant treatment effect compared with placebo, $P < 0.05$; §significant time effect compared with the preimmobilization value.

in the creatine group, compared with the placebo group, the muscle total creatine concentration was higher at the end of the immobilization period, as well as after 3 weeks of rehabilitation ($P < 0.05$). However, along with the declining muscle phosphocreatine levels, muscle total creatine returned to baseline by the end of the study.

DISCUSSION

Our study investigated the impact of creatine supplementation on muscle GLUT4 content and glycogen and total creatine concentrations in healthy subjects during 2 weeks of voluntary leg immobilization followed by 10 weeks of rehabilitation training. Our data are the first to show that creatine supplementation prevents the loss of GLUT4 protein during muscle disuse and increases muscle GLUT4 content above normal levels during subsequent rehabilitation. Furthermore, muscle glycogen concentration was increased during the initial stages of the creatine supplementation.

Glucose transport across the plasma membrane is the rate-limiting step for glucose metabolism. Hence, muscle GLUT4 content is a primary determinant of insulin-stimulated muscle glucose uptake and metabolism (16). Thus, increasing muscle GLUT4 content by transgenic overexpression or by increased contractile activity enhances maximal insulin-stimulated muscle

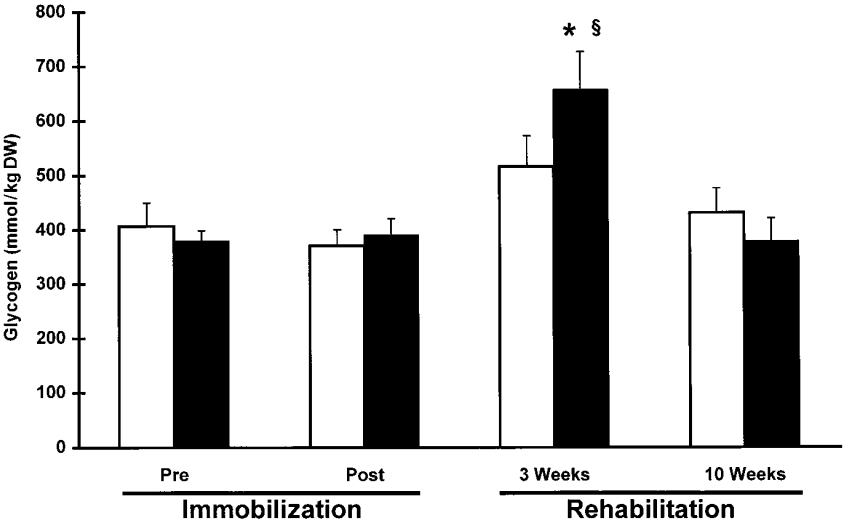

FIG. 2. Effect of creatine supplementation on muscle glycogen concentration during immobilization and subsequent rehabilitation training. Data are means ± SE (n = 8). Muscle samples were taken from the vastus lateralis muscle before and after 2 weeks of immobilization and after 3 and 10 weeks of rehabilitation of the right leg. During immobilization and rehabilitation, subjects ingested creatine monohydrate (■) or placebo (□). See RESEARCH DESIGN AND METHODS for further details. *Significant treatment effect compared with placebo, $P < 0.05$; §significant time effect compared with the preimmobilization value.

TABLE 1
Effect of creatine supplementation on muscle creatine content during immobilization and subsequent rehabilitation training

	Before immobilization	After immobilization	3 weeks of rehabilitation	10 weeks of rehabilitation
Free creatine (mmol/kg DW)				
Placebo	31.3 ± 3.3	41.3 ± 3.6*	43.5 ± 5.4*	37.7 ± 2.9
Creatine	30.6 ± 2.9	48.5 ± 4.5*	53.9 ± 5.4*	43.4 ± 4.0
Phosphocreatine (mmol/kg DW)				
Placebo	76.5 ± 1.8	64.9 ± 3.1*	73.8 ± 2.6	71.6 ± 2.2
Creatine	82.4 ± 6.2	80.2 ± 5.8†	89.7 ± 6.8†	75.1 ± 6.3
Total creatine (mmol/kg DW)				
Placebo	108.8 ± 2.8	106.2 ± 5.7	117.3 ± 5.1	109.3 ± 3.4
Creatine	113.9 ± 8.4	128.7 ± 9.9*†	143.6 ± 11.6*†	118.5 ± 8.0

Data are means ± SE of eight observations and represent concentrations measured in needle biopsy samples obtained from vastus lateralis muscle. Total creatine concentration was calculated as the sum of free creatine and phosphocreatine concentrations measured. Immobilization and rehabilitation procedures are described in RESEARCH DESIGN AND METHODS. *Significant time-effect compared with the preimmobilization value, $P < 0.05$; †significant treatment effect compared with placebo, $P < 0.05$.

glucose uptake. Conversely, reducing the content of GLUT4 by GLUT4 knockout, denervation, or aging impairs insulin-mediated muscle glucose uptake (19). Our data, therefore, suggest that creatine supplementation in humans may increase insulin sensitivity by increasing muscle GLUT4 content.

Over the last decade, substantial evidence has accumulated to show that endurance exercise training elevates muscle GLUT4 content and insulin-stimulated glucose uptake in both healthy (17,20–28) and insulin-resistant muscles (29,30). In this respect, the current study shows that in healthy individuals, a low volume (3 weekly sessions) of moderate resistance training (60% of 1 repetition maximum [RM]), in contrast with endurance training (23–26, 28) or daily maximal resistance training (31), is not a sufficient stimulus to increase muscle GLUT4 content. Ten weeks of rehabilitation training per se did not increase muscle GLUT4 content above the baseline level (Fig. 1). However, the same training regimen in conjunction with oral creatine supplementation resulted in a marked increase of muscle GLUT4 protein content. In fact, our observations indicate that oral creatine supplementation can probably increase GLUT4 protein content in skeletal musculature independent of exercise training. In keeping with earlier observations (17,20–22,31,32), muscle deconditioning by immobilization in the placebo subjects reduced GLUT4 protein content (~20%). Nevertheless, at the end of the immobilization period, GLUT4 content in the creatine group tended to increase by ~10%, which resulted in a 30% difference in muscle GLUT4 between placebo and creatine supplementation in the absence of a training stsore, it is reasonable to conclude that creatine supplementation can increase GLUT4 protein content in human musculature during episodes of either reduced or increased physical activity.

Based on the current knowledge, it is difficult to reveal the molecular basis for the increase in muscle GLUT4 content that occurs during creatine supplementation. It has recently been observed in rats that short-term administration of aminoimidazole-4-carboximide riboside, an AMP-activated protein kinase (AMPK) agonist, increases muscle GLUT4 content (33). Creatine administration that increases AMPK activity by decreasing the phosphocreatine-to-creatine ratio (34) may, thus, explain the increase in GLUT4 protein content in the creatine group. And yet, in both groups the phosphocreatine-to-creatine ratio decreased to the same degree during immobilization and remained below the baseline value during the subsequent rehabilitation period. Furthermore, it has recently been shown that the creatine kinase (CK) and AMPK enzymes colocalize in muscle cells (34). According to the prevailing opinion, in skeletal muscle, such coupling should serve to suppress muscle AMPK activity by maintaining high local ATP:AMP and phosphocreatine-to-creatine ratios in conditions of cellular stress, such as contractions (35). If anything, this inhibitory action is enhanced by the increased muscle phosphocreatine concentration established during the creatine supplementation (Table 1). Thus, evidence for a possible creatine-induced increase in AMPK activity has not been found. Alternatively, there is substantial evidence to suggest that cellular hydration status is an important factor controlling cellular protein turnover (36), which in muscle cells, excluding the contractile proteins, may involve other proteins important to energy homeostasis, such as GLUT4. Creatine is cotransported with Na ions across the sarcolemma, which initiates influx of Cl^- and water to balance electroneutrality and osmolality (11). The resulting increase of cell volume may, in turn, act as an anabolic proliferative signal, which involves activation of the mitogen-activated protein kinase (MAPK) signaling cascade that plays a pivotal role in muscle protein synthesis regulation (37,38). It is warranted to further explore the possible role of intracellular creatine content in modulating the concerted actions of CK, AMPK, and MAPK in regulating GLUT4 synthesis and degradation in muscle cells.

The bulk of glucose in the human body is stored as muscle glycogen. The presence of a high muscle glycogen concentration, in general, indicates adequate insulin stimulation of muscle glucose uptake and glycogen synthesis. Furthermore, a high muscle glycogen concentration is a prerequisite for optimal endurance exercise performance (39). Robinson et al. (8) have recently demonstrated that carbohydrate intake in conjunction with creatine supplementation resulted in greater postexercise muscle glycogen resynthesis than carbohydrate intake alone. Accordingly, in the current study, during the initial 3 weeks of rehabilitation training, muscle glycogen concentration increased by ~30% in the placebo group, whereas a threefold greater increase occurred in the creatine group. This higher-than-average glycogen level,

established by creatine supplementation (>650 mmol/kg DW) (Fig. 2), corresponds with common glycogen levels in young healthy subjects after glycogen "supercompensation" (39). Given that no dietary instructions were administered to the subjects, our findings suggest that the addition of creatine supplementation to a standard diet may eventually result in a postexercise increment of muscle glycogen concentration similar to that found after a classical carbohydrate-enriched glycogen supercompensation dietary protocol (39). Interestingly, after 5 weeks of creatine supplementation, the increase of muscle glycogen content vanished, despite continued creatine supplementation. In fact, during both immobilization and rehabilitation, the pattern of muscle glycogen changes closely mimicked the fluctuations of muscle total creatine content (Table 1) (Fig. 2). In this respect, Low et al. (40) have provided clear evidence that osmotic swelling of muscle cells is a potent stimulus to muscle glycogen synthesis. The 30 mmol/kg DW increase of muscle total creatine, established after 3 weeks of training in the creatine group, was therefore probably sufficient to induce a degree of cell swelling necessary to enhance insulin-stimulated glycogen synthesis (40,36). If such an osmotic trigger mechanism indeed regulates insulin action on glycogen synthesis during creatine supplementation, then the decrease in muscle creatine content beyond 3 weeks of training might also explain the concurrent decrease in the muscle glycogen storage. The mechanism behind the decrease in muscle creatine content during the final stage of the study, despite continued creatine ingestion at a rate presumed to be sufficient for maintaining an elevated muscle creatine content (5 g/day), is unclear (2,41). Studies in rats have demonstrated that long-term high-dose creatine feeding induces a downregulation of muscle total Na-creatine cotransporter protein content (42). In addition, the low creatine transporter content in failing human myocardium has been found to be associated with a decrease in intracellular creatine storage (43).

In conclusion, the current findings provide strong evidence to suggest that *1*) oral creatine supplementation can offset the decline of muscle GLUT4 protein content in skeletal musculature during disuse atrophy, and *2*) oral creatine supplementation increases GLUT4 content during subsequent rehabilitation training. Based on the present findings, it is warranted to evaluate the potential of long-term creatine supplementation as a strategy to prevent or treat disease conditions characterized by peripheral insulin resistance.

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